Simultaneous indirect activity measurements of GH and PRL genes in the same, living mammosomatotrope


ALMOST A DECADE AND A HALF AGO, our group reported that individual, living cells from normal (nontransformed) pituitary tissue could secrete growth hormone (GH) and prolactin (PRL) at the same time (8). This novel finding, obtained through use of reverse hemoletic plaque assays, was confirmed by others who employed either similar methodology or different techniques to demonstrate the presence in the same cell of both hormones (immunocytochemistry [ICC]) (10, 13) or mRNAs (in situ hybridization cytochemistry) (5, 12). Subsequent experimentation with a broad spectrum of mammalian species revealed that this bihormonal “mammosomatotrope” is a constituent cell type of the pituitary gland and that it serves as a transitional cell for the functional interconversions of monohormonal somatotropes and mammotropes that occur during pituitary development and in response to dramatic changes in physiological status (7, 14).

One can envision at least two reasonable explanations to account for the existence of mammosomatotropes. The first and simplest explanation is that a monohormonal cell expressing either the GH or PRL gene would be induced to transcribe, for a time, both of these genes. Eventually, one of these genes would “turn-off” as the cell transdifferentiated to one or the other monohormonal state. In the second scenario, the expression of both genes would be mutually exclusive, as appears to be the case for traditional somatotropes and mammotropes. Concurrent detection of bihormonal storage and release might, therefore, simply reflect the fact that labeled secretory granules can persist intracellularly for many hours or even days after biosynthesis. Likewise, the relatively long half-lives of the GH and PRL mRNAs would favor their dual localization long after cessation of the corresponding transcriptional events. Thus the mammosomatotrope could be more a perception than a reality.

The goal of the present study was to resolve this mammosomatotrope dilemma by assessing transcriptional potential for both the GH and PRL genes in the same living cell. To this end, we adopted an experimental strategy used previously by our group to make real-time measurements of PRL gene expression in single mammotropes (3). This involved transfecting mammotropes with a PRL promoter-driven luciferase reporter construct and at a later time quantifying photonic emissions after exposure to the substrate luciferin. [That the rate of these emissions provides a reliable index of endogenous gene expression is evidenced by observations that agents known to transcriptionally activate (epidermal growth factor) and inhibit (dopamine) the PRL gene in entire populations of cells exert predictable effects on the rate of photonic emissions (3)]. Pursuit of our current objective necessitated expansion of this basic model to enable indirect activity measurements of two different genes at roughly the same time. Here we report how we developed such a system and used it to localize both PRL and GH gene expression to the same, living mammosomatotrope.

MATERIALS AND METHODS

Plasmid cloning strategies. Our first step was to develop a system that would enable activity measurements of two different reporters in the same living cell. For this purpose,
we exploited the published substrate specificities of firefly and Renilla luciferases. We reasoned that, for developmental purposes, these constructs should be driven not only by the same promoter, but also by one not likely to be activated differentially by mono- or bihormonal adiphilins. Accordingly, we prepared constructs in which the coding sequences for firefly and Renilla luciferases were placed under the control of the same cytomegalovirus (CMV) regulatory sequences. In preliminary studies, we found that the promoterless Renilla reporter (pRL-null vector; Promega, Madison, WI) exhibited spurious background photonic activity that could be mistaken as a false positive. Accordingly, we sought first to eliminate this activity by cloning the Renilla-LUC coding sequence (and associated promoters) into the promoterless pGL2-Basic expression vector (Promega), shown previously to lack such background activity. Briefly, the CMV-promoter and Renilla-LUC were PCR amplified from the pRL-CMV-Renilla expression vector (Promega) with primers encoding Kpn I and Xho I ends. The CMV-Renilla PCR product was then subcloned (Kpn I, Xho I) into the pGL2-Basic vector after the removal (Hind III, EcoRI, Klenow fill-in and religation) of the firefly-LUC gene. This construct will be referred to throughout as CMV-Renilla-LUC. As a promoterless control, the CMV promoter was removed from CMV-Renilla-LUC with Sma I, Nhe I restriction endonucleases, and the remaining construct was blunt-ended and religated (i.e., null-pGL2-Renilla-LUC). The CMV-firefly-LUC vector was kindly provided by D. Kurtz (Medical Univ. of South Carolina, Charleston, SC). A promoterless control for the CMV-firefly-LUC was made by Sma I, Nhe I cuts, followed by blunt-ending of the Nhe I site and religation (i.e., null-firefly-LUC). Experiments using the promoterless null-firefly-LUC and null-pGL2-Renilla-LUC reporters revealed no photonic activity above background levels (data not shown). A PRL-Renilla-LUC expression vector was then generated by substituting a 2.5-kbp PRL promoter (pPRL-LUC; R. Maurer, Oregon Health Sciences University, Portland, OR) for the CMV promoter in CMV-Renilla-LUC. The 237-bp GH-firefly-LUC reporter was constructed as reported previously (9).

Development of a dual gene expression system in single, living cells. Monolayered anterior pituitary cells from primiparous lactating rats (days 6–10 of lactation) were plated on photoengraved, gridded, polyl-lysine (GIBCO, Grand Island, NY)-coated, glass coverslips (70,000 cells/cover slips) and cultured in DMEM (GIBCO) + 10% fetal bovine serum (GIBCO) for 48 h. Cells within a grid on a coverslip were then transfected by microinjection with the CMV-firefly-LUC or CMV-Renilla-LUC (1 µg/ml each) or a combination thereof. After microinjection, cells were cultured for an additional 24 h before quantification of photonic activity by use of a VIM photon-counting camera and Argus-50 Image processor (Hamamatsu Photonics Systems, Bridgewater, NJ).

On the day of imaging, cells transfected with CMV-firefly-LUC were placed in a Sykes-Moore chamber (Belco Glass, Vineland, NJ) containing 0.1 mM luciferin (Sigma Chemical, St. Louis, MO) in DMEM, and photons were accumulated in 2-min bins over a 10-min period. The imaging medium was then replaced with one lacking luciferin. This was accomplished by perfusion (1 ml/min; Harvard Apparatus, Holliston, MA) over a 10-min period. Photonic emissions were again monitored continuously in 2-min bins. Then, coelenterazine (5 µM; Molecular Probes, Eugene, OR) was perfused into the chamber, and photonic emissions were accumulated every 2 min for 10 min. This was followed by perfusion once again with luciferin-containing medium, and photon counting was repeated. A similar reciprocal experiment was conducted with cells transfected with CMV-Renilla-LUC, in which measurement in the presence of 0.1 mM luciferin was followed sequentially by measurements with medium alone or with medium containing coelenterazine. It should be noted that the dual expression paradigm employed here was most efficient when measurements of firefly-LUC were followed by Renilla-LUC. The reason for this is that photonic emissions disappeared within 10 min of luciferin removal, whereas more than an hour was required after coelenterazine was flushed out.

Analysis of GH and PRL gene expression in the same, living pituitary cell. Anterior pituitary tissue from lactating rats was dispersed and cultured on glass coverslips, as described earlier. Forty-eight hours later, cells on a grid were cooinjected with the 237-bp GH promoter fused to the firefly-LUC coding sequence (3 µg/µl) and the 2,500-bp PRL promoter fused to the Renilla-LUC coding sequence (0.75 µg/µl). After 24 h, cells were subjected sequentially to image analysis of photonic activity generated in the presence of the appropriate LUC substrate (GH-firefly-LUC = 3 mM luciferin; PRL-Renilla-LUC = 5 mM coelenterazine). In a typical experiment, luciferin was infused into the Sykes-Moore chamber containing the cells, and photonic activity indicative of GH-mediated gene transcription was recorded. Next, the chamber was flushed with medium devoid of substrate, and this was followed by infusion of coelenterazine for measurement of photonic signals reflective of PRL promoter-driven Renilla-LUC activity.

Post facto ICC was conducted for GH on coverslips (n = 20) obtained from four different experiments. This was done to confirm that expression of the GH-firefly-LUC was restricted to cells containing GH, as had been established previously for PRL (3). Moreover, these data were used to establish whether there was a congruence between GH biosynthesis and gene expression in the same cells. To this end, cells were fixed with B5-buffered Formalin for 45 min immediately after photonic imaging, rinsed, and subjected to ICC identification of GH as reported previously (2).

RESULTS

Substrate specificities permit concurrent, independent measurements of firefly and Renilla luciferases. As shown in Fig. 1, cells microinjected with only the CMV-firefly-LUC reporter exhibited considerable photonic activity in the presence of luciferin. This activity decreased to negligible levels within 10 min of luciferin removal, and subsequent exposure to coelenterazine did not evoke an increase in photonic activity. In contrast, reinfusion of luciferin increased the rate of photonic emissions to a level indistinguishable from that observed in response to the initial luciferin challenge. Similar substrate specificity was observed when cells were transfected solely with the CMV-Renilla-LUC construct (Fig. 1, inset). Next, we cotransfected individual pituitary cells (n = 80) by microinjection with both the CMV-Renilla-LUC and CMV-firefly-LUC reporters and quantified photonic emissions after sequential additions of luciferin and coelenterazine. We found that transfected cells emitted 104.6 ± 13.4 (SE) and 444.0 ± 28.6 specific photonic emissions/min for CMV-firefly-LUC and CMV-Renilla-LUC, respectively. Complete separation of the firefly and Renilla signals was evident during the intervening wash phase, at which time only 1.5 ± 0.27 specific photonic emissions/min were recorded (i.e., background levels) before
coelenterazine infusion. Clearly, these data demonstrate the potential for independent monitoring of promoter activity for two different genes within the same, single, living cell.

Use of dual reporter system reveals pituitary cells that concurrently express the GH and PRL genes. Having established that we could independently monitor the activity of two different reporter constructs in the same cell, we next modified this system to enable concurrent, independent assessments of GH and PRL gene expression. Specifically, individual pituitary cells were microinjected with a GH promoter-driven firefly luciferase reporter construct or a PRL promoter-driven Renilla luciferase construct. After exposure to respective substrates, we found (Table 1) that we could detect GH or PRL gene expression within individual pituitary cells in primary culture. Activity measurements after cotransfection of both reporters revealed cells capable of expressing both genes concurrently (Fig. 2 and Table 1). At first glance, these dual expressers appeared to be quite rare in that they accounted for only 15 cells out of 355 that expressed GH or PRL. However, a more rigorous analysis suggests that we grossly underestimated the size of the GH-expressing population. This view is supported by our observation that 23.7 ± 3.1% of pituitary cells stained positively for GH in our experiments, whereas photonic emissions could be detected in only 2.0 ± 0.43% of cells transfected with the GH-firefly-LUC reporter plasmid. What might account for such a discrepancy? One possibility is that the GH promoter is much weaker than its PRL counterpart in the context of the model we used for these experiments. Consistent with this notion is our finding that the activity of PRL-Renilla-LUC was more than 50-fold higher than

Table 1. Photonic activity of cells microinjected with GH and/or PRL promoter-driven reporters

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<tr>
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<th>GH-firefly-LUC</th>
<th>PRL-Renilla-LUC</th>
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<tr>
<td>Microinjection of only one reporter*</td>
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<tr>
<td>GH expressing cells (10)</td>
<td>5.7 ± .9</td>
<td>117.1 ± 11.9</td>
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<tr>
<td>PRL expressing cells (32)</td>
<td></td>
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<tr>
<td>Microinjection of both GH and PRL reporters†</td>
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<tr>
<td>GH-only cells (3)</td>
<td>5.7 ± 1.1</td>
<td>1.4 ± 0.8‡</td>
</tr>
<tr>
<td>PRL-only cells (337)</td>
<td>0.39 ± 0.04‡</td>
<td>138.6 ± 9.2‡</td>
</tr>
<tr>
<td>GH &amp; PRL expressers (15)</td>
<td>4.7 ± 1.2</td>
<td>256.7 ± 53.1</td>
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Experiments were carried out as in Figure 2. Values are (means ± SE) specific photonic emissions/min (i.e., signal-background), and nos. of observations are in parentheses. GH, growth hormone; PRL, prolactin; LUC, luciferase. *Cells were transfected in separate experiments with either the GH-firefly-LUC or PRL-Renilla-LUC reporters, and photonic measurements were acquired in the presence of the appropriate substrate. †GH- and PRL-only cells are those cells that were cotransfected with both GH and PRL reporters but exhibited only GH or PRL promoter-driven reporter activity, respectively. GH & PRL expressers are those cells that were cotransfected with both gene reporters and identified as expressing both genes. ‡These values are not significantly different from background (P > 0.1).
that of the GH-firefly-LUC reporter in coexpressing cells (Table 1). It is also noteworthy that the photonic potential of Renilla-LUC is acknowledged by others to be stronger than that of firefly-LUC (1), and our observation that CMV-Renilla-LUC provided a 4.2-fold stronger signal than CMV-firefly-LUC is consistent with this idea. Finally, it is possible in cells cotransfected with both plasmids that one of them may have suppressed activity of the other, as has been reported previously (6), thereby further affecting our sensitivity of detection. These sensitivity considerations notwithstanding, the majority of cells positive for GH gene expression (83.3%) were also found to be positive for PRL gene expression.

**DISCUSSION**

Dual gene reporter systems for use in whole populations of cells (1) as well as single cells (4, 11) have been developed previously. Invariably, the second reporter (under control of a viral promoter) has been used to assess transfection efficiency and/or to normalize the relative activity of the first "physiologic" reporter. Here, we describe for the first time a method for indirectly quantifying the activity of two physiologically relevant genes in the same living cell. Unlike previous systems, this one can achieve complete separation of reporter signals, thereby obviating concerns about false positive results. Additional advantages of the current system are that the activities of both genes can be assessed within a reasonable time frame (20 min) and that the procedures involved do not compromise cell viability. This latter advantage raises the possibility that two functionally interrelated genes can be monitored, in the same cell, multiple times over several hours or even days, as has been achieved previously with a single gene reporter system (3, 15).

With this powerful tool in hand, we set out to answer the compelling question posed earlier: can the GH and PRL genes be transcribed concurrently in an individual, living cell? The answer to this question is an unequivocal yes. Although the size of the GH-expressing population was admittedly underestimated because of sensitivity considerations, we did find that the vast majority of detectable GH expressers were also positive for PRL. Should this pattern of overlap persist when these sensitivity problems are overcome, our findings would suggest that many if not most acidophils transcribe both genes at the same time. Although a particular cell might transcribe primarily the PRL gene at a given time, the "pilot light" for GH transcription would continue to flicker. Such a possibility was first raised by Hashimoto et al. (10) who employed dual-labeling, colloidal gold, electron-microscopic ICC to show that most acidophils in the bovine pituitary were either conspicuously mammotrop or somatotrop. However, most cells stored significant quantities of the minority hormone as revealed by objective, quantitative criteria.

In summary, we have shown clearly that independent measurements of two gene promoters can be achieved within the same living cell. Moreover, we have shown that expression of the GH and PRL genes is not mutually exclusive. Taken together with previous reports on simultaneous storage of GH and PRL mRNAs (5, 12) and proteins (10, 13) and dual secretion (8), our results on concurrent transcription demonstrate that the mammosomatotrope is a bona fide cell type and not just an artifact arising from the potentially long half-lives of stored hormones and corresponding mRNAs.

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